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13. ABSTRACT (Maximum 200 Words)
AKT1 belongs to the AKT/PKB protein-serine kinase family and is the cellular homolog of the v-akt oncogene. Gene amplification and overexpression of AKT are associated with adenocarcionomas of the stomach, breast, ovary, and pancreas. AKT1 is coupled to growth factor-dependent proliferation and resistance to apoptosis, but these processes have not been studied in either mammary epithelial cells or in breast cancer. The broad objectives of this proposal are to determine the role of the AKT1 in the proliferation, survival and transformation of mammary epithelial cells using two transgenic mouse strains in which expression of either AKT1 or a constitutively active form of AKT1 is directed to the mammary gland under the control of a mammary gland-specific promoter. The use of these two transgenic models will allow determination of the role of AKT1 in mammary gland hyperplasia, dysplasia and tumorigenesis, and will address the hypothesis that AKT1 is involved in the growth, survival, and transformation of mammary epithelial cells. This hypothesis will be tested using two experimental approaches. First, transgenic mice will be generated with mammary gland-directed expression of wild-type AKT1 or constitutively active myristoylated AKT1 (myrAKT1) to determine their effects on mammary hyperplasia and tumorigenesis. Second, primary mouse mammary epithelial cells will be transduced with ecotropic retroviruses expressing either AKT1 or myrAKT1 to determine their impact on growth factor-dependent proliferation, susceptibility to apoptosis induced by serum deprivation or the P13K inhibitor, LY294002, as well as transformation determined by anchorage-independent growth in soft agar and tumor formation in nude mice. Gene arrays of suppression subtractive hybridization will be used to identify genes modulated by AKT1 that are involved in its anti-apoptotic and proliferative activities. These studies will determine the role of the potentially oncogenic AKT1 gene that is linked to growth factor activation and inhibition of apoptosis, on the etiology of mammary gland hyperplasia, dysplasian and transformation, and provide a rationale for the development of antiporliferative and chemopreventive therapies for breast cancer.

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INTRODUCTION:

AKT1 (protein kinase B, PKB) is the human cellular protein-serine/threonine kinase homolog of the v-akt oncogene (1, 2) that was rescued from the transforming AKT-8 retrovirus responsible for spontaneous lymphomas in the AKR mouse (3). AKT exists as three closely related subtypes, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ that have 70% homology to the protein kinase C catalytic domain (4). AKT1 is 480 amino acids and encodes a 56 kDa protein that shares 98% homology with murine akt1 and 89% homology with other AKT isoforms. AKT1 maps to chromosome 14q32, the same locus that is rearranged in T-cell lymphomas and leukemias and ataxia telangiectasia (5). AKT2 maps to chromosome 19q13.1-13.2, the region amplified in ovarian carcinoma (6), and AKT3 maps to chromosome 1q43-q44 (7). Gene amplification or overexpression of one or more isoforms of AKT has been noted in human primary tumors of the breast, prostate, ovary and brain (8), where AKT activation is coupled to growth factor-dependent proliferation and resistance to apoptosis (9-12). Growth factors such as IGF-I, PDGF-B, EGF and bFGF as well as the v-Ha-Ras and v-src oncogenes activate AKT1 (13, 14); however, not all growth factor signaling pathways affect AKT1 equally since dominant-negative Ha-Ras abolishes bFGF-dependent AKT1 activation, but partially blocks the effects of PDGF and EGF (15). In addition, Ha-Ras and R-Ras, but not c-Raf, Rho, Rac and Cdc42 stimulate AKT1 activity (16). Growth factor-coupled signaling through phosphatidylinositol 3-kinase (PI3-K) results in AKT activation (17), and PI3-K inhibitors such as wortmannin and LY294002 block this effect (18, 19). Mammary gland oncogenes such as polyoma middle-T antigen, ErbB-2 and a constitutively active EGFR, activate PI3-K (20-22), and hence, maintain AKT in an activated state (23). This also occurs *in vivo* in the mammary gland of mice expressing a constitutively active AKT1 transgene (24-26), as well as in PTEN knockout transgenic mice expressing the Wnt-1 transgene (27).

Cellular stresses such as heat shock and oxygen radicals activate AKT (28) linking it to a survival pathway that is similar to the apoptotic stress response to toxins and anticancer drugs (29). v-Akt protects cells against apoptosis induced by IL-3 withdrawal, etoposide or paclitaxel (30), and overexpression of AKT1 or v-akt prevents apoptosis induced by serum deprivation or wortmannin (9, 31). AKT1 prevents apoptosis mediated by MAP kinase p38 and caspase-3 inhibition (32), and v-akt protects cells from apoptosis (anoikis) induced by detachment from the extracellular matrix (33). AKT1 activation is associated with integrin-mediated adhesion in platelets and fibroblasts (34), but not with membrane ruffling or lamellopodia formation (15).

AKT is involved in the post-translational regulation of many proteins through the ubiquitination/proteasome pathway. GSK-3 is a substrate and downstream effector of AKT (35) and is inactivated upon phosphorylation. Inhibition of GSK-3 leads to greater resistance to proteasomal degradation for β-catenin, which enhances tumor cell growth and invasion (36, 37). GSK3 also inhibits c-jun, and therefore, GSK3 inhibition by AKT would be expected to increase transcription factor AP-1 activity (38) leading to activation of matrix metalloproteases (MMPs) (39). The seminal finding that AKT1 and v-akt activate matrix metalloproteinase-2 (MMP-2) in mammary epithelial cells by increasing its stability to proteosomal degradation provides a basis for extracellular matrix invasion (40).

The purpose of this proposal was to investigate the role of AKT1 in mammary tumorigenesis and transformation by the generation of two transgenic mouse models, as well as in such processes as transformation and invasion, where its role has not been well-defined.

BODY:

Statement of Work:

Year 1: [note Tasks 3-8 were incorrectly numbered Task 4-9]

1. Backcross AKT1 mice to develop pure transgenic strains. Determine histopathological changes and mammary tumor formation in nulliparous and multiparous AKT1 mice. Characterize mammary gland expression of AKT1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays.

2. Begin pronucleus injections with the MMTV/Gag-akt1 plasmid and generate founder mice.

3. Evaluate Gag-akt1 founder mice for mammary gland expression of Gag-akt1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays. Characterize mammary gland morphology and histopathology in progeny from Gag-akt1 mice.

4. Prepare AKT1, AKT1K179E and Gag-akt1 retroviruses.

5. Transduce non-transgenic primary mouse mammary epithelial cells with AKT1 and Gag-akt1 retroviruses and determine their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

6. Test AKT1- and Gag-akt1-transduced primary epithelial cells from non-transgenic mice for their apoptotic response to the PI3K inhibitor, LY294002, by measuring DNA fragmentation by *in situ* end-labeling.

7. Test primary mammary epithelial cells from AKT1 mice for their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

8. Test primary mammary epithelial cells from AKT1 mice for their apoptotic response to the PI3K inhibitor, LY294002, and to the dominant-negative AKT1K179E, by measuring DNA fragmentation by *in situ* end-labeling.

Results:

1. Characterization of MMTV-AKT1 transgenic mouse was completed and the results published (see Appendix for reprint) (26). These mice did not exhibit mammary tumors over their lifespan, but did show marked mammary gland hyperplasia and suppression of mammary gland involution following cessation of lactation, indicating a block in mammary epithelial cell apoptosis. This was associated with elevation of cyclin D1 and phosphorylation of the proapoptotic protein, BAD, which results in its inactivation (26).
2. Pronuclear injections to generate MMTV-Gag-akt1 mice were completed.
3. Generation of MMTV-Gag-akt1 mice was not completed since founder MMTV-Gag-akt1 mice were unable to be produced, suggesting that this transgene was embryonic lethal. As an alternative approach, the ovine β-lactoglobulin (BLG) promoter (provided by Dr. Bruce Whitelaw, Roslin Institute, Edinburgh, Scotland) was used to express a constitutively active myristoylated form of AKT1. The BLG promoter is strongly activated in alveolar secretory cells only during late pregnancy and lactation. Founder mice expressing a constitutively active AKT1 gene under the control of the BLG promoter were produced and two founder lines are being characterized for mammary gland morphology and transgene expression by western blotting and IHC. Since the BLG promoter is only activate during late pregnancy and lactation, characterization can only be done in multiparous mice, thereby necessitating a considerably longer time for analysis.
4. Preparation of Akt1, Akt1K179E and Gag-Akt1 retroviruses was completed.
5. Mouse mammary epithelial cell lineCOMMA-1D derived from mammary epithelial cells from Balb/c mice was transduced with retroviruses expressing AKT1, AKT179E and Gag-akt1 to complete this task. This cell line, rather than primary epithelial cells, was used for consistency and reproducibility since primary cells were difficult to grow and maintain in culture. Their proliferative response to IGF-1, PDGF-B, EGF and b-FGF was not tested, but no changes in growth were noted in the presence of 10% fetal calf serum.
6. Cell lines from non-transgenic mice were not prepared or tested for their apoptotic response to LY294002
7. Cell lines from MMTV-AKT1 mice were not prepared or tested for their proliferative response to growth factors.
8. Primary cells from MMTV-AKT1 mice were not prepared or tested for their apoptotic response to LY294002.

Other results: COMMA-1D cells expressing AKT1 did not exhibit transformation when grown in soft agar; cells expressing myrAKT1 or v-akt were weakly transforming (see Appendix for reprint) (41). Cells expressing AKT1

were not tumorigenic as isografts in syngeneic Balb/c mice (41). COMMA-1D cells were also engineered to express the avian retrovirus receptor, tva, to make them permissive to infection by avian retroviruses (see Appendix for reprint) (40). Cells expressing AKT1 or v-akt exhibited invasivness in Matrigel and produced matrix metalloprotease-2 (MMP-2), suggesting that extracellular matrix degradation occurred separately from transformation. MCF-7 human breast carcinoma cells were also engineered to express tva and were subsequently transduced with an avian retrovirus expressing the AKT kinase, PDK1, Akt1K179E (kinase-dead) or both PDK1 and AKT1K179E to see if a dominant-negative AKT1 could inhibit PDK1 signaling. Neither cell growth nor apoptosis induced by LY294002 was affected by expression of either PDK1, AKT1K179E or both genes together (unpublished results).

Year 2:

1. Finish evaluating nulliparous and multiparous AKT1 mice for mammary tumor formation.

2. If no tumor formation is observed in AKT1 mice, administer DMBA and determine the incidence and latency of tumor formation between non-transgenic and AKT1 mice.

3. Backcross Gag-akt1 mice to develop pure transgenic strains. Determine histopathology and mammary tumor formation in nulliparous and multiparous Gag-akt1 mice. Characterize mammary gland expression of Gag-akt1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays.

4. Assess gene expression by differential hybridization in AKT1 and Gag-akt1-transduced primary mammary epithelial cells from non-transgenic mice vs. cells transduced with an empty virus. Assess gene expression by suppression subtractive hybridization in AKT1 and Gag-akt1-transduced primary mammary epithelial cells from non-transgenic mice after treatment with LY294002 or vehicle.

5. Test primary mammary epithelial cells from Gag-akt1 mice for their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

6. Test primary mammary epithelial cells from Gag-akt1 mice for their apoptotic response to the PI3K inhibitor, LY294002, and to the dominant-negative AKT1K179E, by measuring DNA fragmentation by *in situ* end-labeling.

Results:

1. MMTV-AKT1 mice did not exhibit mammary tumor formation during their life span.
2. MMTV-AKT1 mice were not tested for their response to DMBA, but we expect to do these studies in the coming year.
3. MMTV-Gag-Akt1 mice were unable to be produced. BLG-myrAkt1 mice are in the process of being evaluated, but no mammary gland expression of myrAkt1 or a significant phenotype has been noted so far in lactating mice.
4. COMMA-1D/Akt1 cells were examined for changes in gene expression using the Mouse Angiogenesis and Mouse Pathway Finder arrays from SuperArray, Inc. Each array contained 96 genes associated with either angiogenesis or multiple signal transduction pathways. The following genes exhibited changes in AKT1-expressing cells vs control cell s transduced with an empty virus: BMP2 [bone morphogenetic protein 2] (+329%), BMP4 [bone morphogenetic protein 4 (+280%), Bcl2 (+423%), Bcl2-like [Genbank L35049] (+423%), En1 [engrailed homolog 1, Genbank U41751] (+186%). Changes in BMP4 mRNA levels could not be confirmed by northern or western blot and were not pursued further.
5. and 6. Not completed since Gag-Akt1 mice were unable to be not produced.

Year 3:

1. Finish evaluating nulliparous and multiparous Gag-akt1 mice for mammary tumor formation.

2. If no tumor formation is observed in Gag-akt1 mice, administer DMBA and determine the incidence and latency of tumor formation between non-transgenic and Gag-akt1 mice.

3. Finish evaluating AKT1 mice for tumor formation following DMBA treatment, if required.

4. Sequence and identify genes that are regulated by AKT1 and Gag-akt1 that were identified by suppression subtractive hybridization. Determine the expression of these genes in primary epithelial cells from non-transgenic and AKT1 and Gag-akt1 transgenic mice, and in response to apoptosis induced by LY294002, serum-deprivation and a dominant-negative AKT1K179E.

6. Evaluate the mammary gland and mammary tumors from AKT1 and gag-akt1 transgenic mice for their expression of genes identified by suppression subtractive hybridization.

Results:

1. and 2. not completed since MMTV-Gag-Akt1 mice could not be produced.
3. DMBA administration to MMTV-Akt1 mice was not initiated since we had to rederive our transgenic line due to a MPV infection outbreak in our animal facility. Frozen sperm from our two founder lines will be used to rederive the transgenic lines.
4. Subtractive hybridization has not been initiated. This will require more manpower and since MMTV-AKT1 transgenic mice did not exhibit a malignant phenotype, we have not commited more time to this task. The results of gene array analysis suggest that AKT1 may regulate Bcl2 mRNA expression and will be addressed in future studies if confirmed by northern analysis.
5. Affymetrix mouse gene array analysis of the mammary gland from MMTV-AKT1 transgenic mice was not initiated since the Cancer Center only recently made this available as a core resource. In addition, MMTV-AKT1 mice need to be rederived, and we expect this task to be completed as soon as this can be accomplished.

Extension Year 4:

Results:

1. Additional AKT isoforms were investigated for their transforming activity *in vitro* to determine if they would be candidates as a transgene for MMTV-directed expression in the mammary gland. RCAS virus encoded wild-type and myristoylated AKT1, AKT2, AKT3 was used to transduce COMMA-1D/tva cells (40), and were not found to be transforming in agreement with our previous results for AKT1 (41). In addition, wild-type and myristoylated forms of the caspase-3-resistant variant, AKT1-D462N (42) was tested by avian retrovuus transduction of COMMA-1D cells and was not found to be transforming.

Conclusion:

1. These results indicate that all AKT isoforms are not transforming in mouse mammary epithelial cells and therefore, are not suitable transgene candidates for expression in the mouse mammary gland.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed and published the characterization of MMTV-AKT1 transgenic mice (see Appendix, Ackler *et al.*).
- Produced BLG-myrAKT1 transgenic founder mice, which are being characterized (unpublished results).
- Completed and published a study showing that COMMA-1D mammary epithelial cell lines made amenable to avian retroviral infection and expressing AKT1or v-akt exhibited increased MMP-2 activity and invasion (see Appendix, Park *et al.*).
- Completed and published a study showing that mammary epithelial cells expressing AKT1 were not transformed *in vitro* or tumorigenic as isografts in syngeneic mice, but that the AKT kinase, PDK1, was

highly tumorigenic (see Appendix, Zeng et al.).

- Using the yeast two-hybrid system, discovered that glutathione peroxidase and a protein of unknown function interact with AKT1 (unpublished results).
- Produced COMMA-1D mammary epithelial cells expressing AKT1 by murine retroviral transduction (see Appendix, Park et al.).
- Produced COMMA-1D mammary epithelial and breast cancer cell lines expressing the avian retrovirus receptor, tva, which are amenable to avian retroviral transduction (see Appendix, Zeng et al.).

REPORTABLE OUTCOMES:

Abstracts:

Ackler, S., Ahmad, S., Tobias, C., Johnson, M. and Glazer, R.I. The development of mammary hyperplasia in MMTV-AKT1 transgenic mice. Proc. Amer. Assoc. Cancer Res. 41:714, 2000 (S. Ackler chosen for a Young Investigator Award)

Ackler, S., Ahmad, S., Johnson, M. and Glazer, R.I. Delayed involution in MMTV-c-AKT transgenic mice. Mouse Models of Cancer, La Jolla, CA, November 29-December 3, 2000.

Park, B-K, Zeng, X. and Glazer, R.I. AKT1 induces invasion on Matrigel and MMP-2 activity in mouse mammary epithelial cells. Proc. Amer. Assoc. Cancer Res. 42:16, 2001. (B-K Park chosen for a Young Investigator Award).

Zeng, X., Xu, H. and Glazer, R.I. 3-Phosphoinositide-dependent protein kinase1 (PDK1) transforms mouse mammary epithelial cells: association with enhanced expression of protein kinase Cα and activation of protein kinase Cα and AKT1. Proc. Amer. Assoc. Cancer Res. 43:375, 2002.

Xie, Z., Zeng, X. and Glazer, R.I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with increased c-Myc expression and downregulation of caveolin-1. Proc. Amer. Assoc. Cancer Res. 44:1426, 2003

Papers:

Park, B.-K., Zeng, X. and Glazer, R.I. Induction of MMP-2 and invasion in mammary epithelial cells expressing AKT1. Cancer Res. 61:7647-7653, 2001.

Ackler, S., Ahmad, S., Tobias, C., Johnson, M. and Glazer, R.I. Delayed involution and mammary hyperplasia in MMTV-AKT1 transgenic mice. Oncogene 21:198-206, 2002.

Zeng, X., Xu, H., Park, B.-K., and Glazer, R. I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Cα. Cancer Res. 62:3538-3543, 2002.

Xie, Z., Zeng, X., Waldman, T. and Glazer, R.I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) activates β-catenin and c-Myc expression and downregulates caveolin-1. Cancer Res. 63:5370-5375, 2003

Cell lines. COMMA-1D/tva, COM/AKT1, COMMA-1D/v-akt1, COMMA-1D/myrAKT1 and COMMA-1D/PDK1 mouse mammary epithelial cell lines; MCF-7/tva, MCF-7/PDK1 and MCF-7/dnAKT1 human breast carcinoma cell lines.

Animal models.

MMTV-AKT1 (completed) and BLG-myrAKT1transgenic mice (in progress)

CONCLUSIONS:

We have realized most of our goals for the third year, including generation of a new transgenic mouse, and have published three papers describing these results. The second transgenic mouse model, BLG-myrAKT1, is expected to be completed this year. Using the yeast two-hybrid system, we have identified two gene products not previously known to interact with AKT1, cellular glutathione peroxidase, and an EST that is highly expressed in several primary

human cancers, but is as yet undefined. Neither the wild-type or myristoylated versions of the three AKT isoforms nor the wild-type or myristoylated caspase-3-resistant variant AKT1-D462N were transforming in mouse mammary epithelial cell line COMMA-1D.

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Robert I. Glazer, Ph.D., P.I., 10%
Shakeel Ahmad,, Ph.D., Res. Asst. Prof., 10%
Xiao Zeng, Ph.D., Res. Assoc., 30%
Zhihui Xie, Ph.D., Res. Assoc., 10%

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Transformation of Mammary Epithelial Cells by 3-Phosphoinositide-dependent Protein Kinase-1 (PDK1) Is Associated with the Induction of Protein Kinase Cα[1]

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ABSTRACT

3-Phosphoinositide-dependent protein kinase 1 (PDK1) is a mediator of multiple signaling pathways coupled to growth factor receptor activation in human cancers. To evaluate the role of PDK1 in mammary gland oncogenesis, COMMA-1D mouse mammary epithelial cells were retrovirally transduced with PDK1, and transformation was measured by anchorage-independent growth in soft agar. PDK1-expressing cells exhibited a high degree of transformation that was associated with the activation of Akt1 and an elevation of protein kinase Cα (PKCα) expression. Cells overexpressing Akt1 did not exhibit anchorage-independent growth, whereas PKCα overexpression produced significant transformation, although to a lesser extent compared with PDK1. Coexpression of Akt1 and PKCα led to a more than additive effect on transformation activity. Isografts of either PDK1- or PKCα-expressing cells but not Akt1-expressing cells in syngeneic mice led to formation of poorly differentiated mammary carcinomas. PDK1 was highly expressed in a majority of human breast cancer cell lines. These results suggest that activation of PDK1 can lead to mammary tumorigenesis, in part through PKCα, and that PDK1 expression may be an important target in human breast cancer.

INTRODUCTION

The deregulation of protein kinases involved in modulating cell proliferation and survival is often associated with malignant transformation (1). One pathway that prominently plays a role in oncogenesis is the growth factor receptor-coupled activation of PI3-K[3] (2) and its central effector, PDK1 (3, 4). PDK1 was originally identified on the basis of its ability to phosphorylate and activate Akt at Thr308 (5–9). Phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, generated by PI3-K at the plasma membrane, direct membrane localization of PDK1 through its pleckstrin homology domain (10) resulting in the autophosphorylation of PDK1 at Ser241 within the activation loop (7). Activation of PDK1, in turn, effects transphosphorylation within the activation loop of several protein Ser/Thr kinase families, including AKT (5), PKC (11–15), ribosomal p90 S6 kinase, S6K (16–19), and SGK (20, 21). In addition, PDK1 activates the Rho kinase, PKN/PKC-related kinase 1, and the Rac/Cdc42 kinase, PAK1 (22–24), which are associated with increased invasion and metastasis (25). This suggests that PDK1 functions as a master control point for the activation of a number of signaling pathways involved in proliferation, survival, and invasion.

There are few studies of the functional role of PDK1 in cancer cells. Recently, it was found that antisense oligonucleotides targeting PDK1 could block the proliferation of U-87 glioblastoma cells by promoting apoptosis (26). This effect could have resulted from inhibition of one or more downstream targets of PDK1 such as Akt or PKC. The PDK1 substrates, Akt1, Akt2, and Akt3, are highly expressed in several human cancers (27), and one or more PKC isoforms are elevated in breast and colon tumors (28, 29). Akt2 and PKCα have also been shown to transform rodent fibroblasts *in vitro*, whereas Akt1 and Akt3 were not oncogenic (30, 31). Despite these findings, a direct functional link between PDK1 activation and processes involved in malignant transformation has not been explored.

In the present study, we demonstrate for the first time that expression of PDK1 in mouse mammary epithelial cells is sufficient to elicit transformation *in vitro* and tumor formation *in vivo*. This process was associated with activation of Akt1 and PKCα, as well as an increase in PKCα levels, and expression of PKCα but not Akt1 in mammary epithelial cells resulted in transformation *in vitro* and tumorigenesis *in vivo*. These results suggest that the PKCα signaling pathway is a major route mediating PDK1-dependent transformation.

MATERIALS AND METHODS

Cells and Antibodies. Mouse mammary epithelial cell line COMMA-1D (Ref. 32; provided by Dr. Robert Dickson, Georgetown University) was maintained at 37°C under 5% CO_2 in IMEM with 1× supplement (2.5% fetal bovine serum, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin). Cell pellets of human breast cancer cell lines MCF-7, ZR-75–1, T47D, MDA-MB-231, MDA-MB-436, MDA-MB-157, BT483, and SK-Br-3, and human mammary epithelial cell line MCF-10A were obtained from the Lombardi Cancer Center Tissue Culture Core Facility. Polyclonal antibodies to PDK1, Akt1, Akt1/pThr308, PKCα, pSer657PKCα, PKCζ, SGK, S6K, and pThr412S6K, and monoclonal antibodies to the myc epitope tag (9E10) were obtained from Upstate Biotechnology, Inc (Lake Placid, NY). Monoclonal antibodies to PKCβ, δ, ε, η, θ, and μ were obtained from Transduction Laboratories (Lexington, KY) and to β-actin (C-11) from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Construction, Virus Production, and Transduction of COMMA-1D cells. The human PDK1 cDNA with an NH_2-terminal myc epitope tag was provided by Dr. Dario Alessi, University of Dundee, Dundee, United Kingdom. The mouse Akt1 cDNA was obtained from Drs. Peter Vogt and Masashiro Aoki, The Scripps Research Institute, La Jolla, CA. Rabbit PKCα cDNA was provided by Dr. Shigeo Ohno, Yokohama University, Yokohama, Japan. Akt1 and PKCα were amplified by PCR and subcloned into vectors pGADT7 and pGBKT7 (Clontech, Inc.), respectively, to obtain an NH_2-terminal hemaglutinin epitope tag for Akt1 and an NH_2-terminal myc epitope tag for PKCα. All of the cDNAs were then cloned into the retroviral vector pSRαMSVtkneo (33). Retroviral vector pCMV/hyg was generated by replacement of the Tet on/off control element on vector pRevTRE (Clontech, Inc) with the CMV promoter from pRc/CMV (Invitrogen). Either pSRαMSVtkneo or pSRαMSVtkneo encoding PDK1, Akt1, or PKCα were cotransfected with the pSV-ψ-E-MLV ecotropic vector into 293T cells. After 48 h, the supernatants were collected, mixed with an equal volume of fresh IMEM medium plus 2× supplement in the presence of 4 μg/ml Polybrene, and added to COMMA-1D cells. After four rounds of infection, G418-resistant COMMA-1D cells were selected for 2 weeks. To generate cells coexpressing Akt1+PKCα, Akt1+PDK1, or PKCα+PDK1, a second round of transduction was carried

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[3] The abbreviations used are: PI3-K, phosphoinositide 3-kinase; Akt1, protein kinase Bα; IGF-I, insulin-like growth factor-1; IMEM, Improved Minimal Essential Medium; PDK, 3-phosphoinositide-dependent protein kinase; CMV, cytomegalovirus; PKC, protein kinase C; S6K, ribosomal p70 S6 kinase-1; SGK, serum- and glucocorticoid-regulated kinase.

out using amphotropic viruses produced in 293T cells cotransfected with pCMV/hyg-Akt1 and the pSV-ψ-A-MLV amphotropic vector. After 2 weeks of selection in 50 μg/ml hygromycin, the expression of both genes was confirmed by Western blotting.

Western Analysis for pThr308Akt1, pSer657PKCα, and pThr412S6K. Subconfluent cells were lysed in Buffer A containing: 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerol phosphate, 5 mM Na PP_i, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 1 μM Microcystin LR, and protease inhibitor mixture (Boehringer-Mannheim). Whole cell extracts containing 50 μg of protein were analyzed by SDS-PAGE and Western blotting. To measure IGF-I-dependent activation of Akt1 and PKCα, cells were grown to subconfluence, washed once in serum-free IMEM medium, and incubated overnight (12–14 h) in serum-free medium. Fresh serum-free medium was added with or without 100 ng/ml IGF-I, and cells were incubated for 10 min at 37°C. Stimulation was stopped by the addition of serum-free medium prechilled to 4°C. Cells were collected on ice by scraping into prechilled Buffer A. Lysates were clarified by centrifugation at 13,000 × *g* for 15 min. Lysates (400 μg protein) were then incubated at 4°C for 2 h with 4 μg of prewashed protein A/G-agarose (Santa Cruz Biotechnology) adsorbed with anti-Akt1, anti-PKCα, or anti-S6K antibodies. After washing four times in Buffer A, bound proteins were eluted directly into Laemmli sample buffer by boiling for 5 min. The eluted proteins were then analyzed by Western blotting using the antibodies specific for pThr308Akt1, pSer657PKCα, and pThr412S6K.

PDK1 Kinase Assay. Immunoprecipitation was carried out as described above with 4 μg of anti-myc IgG and 400 μg protein of whole cell lysate. PDK1 activity was measured with the PDK1 Kinase Assay kit (Upstate Biotechnologies, Inc.) according to the manufacturer's instructions. This assay measures PDK1-dependent phosphorylation and activation of recombinant SGK and the subsequent incorporation of [^{32}P]ATP into a basic peptide substrate.

Soft Agar Assay for Anchorage-independent Growth. After eight passages of the retrovirally transduced cells, exponentially growing cells (1×10^5) were suspended in 2 ml 0.33% (w/v) SeaPlaque agar (BMA, Rockland, ME) dissolved in IMEM containing 1× supplement and layered over 1% agar dissolved in IMEM containing 1× supplement in six-well plates. Cultures (in triplicate for each condition) were fed with fresh medium once a week. Colonies >50 cells were counted visually at the end of 3 weeks.

Isograft Transplantation into Syngeneic Mice. COMMA-1D cells were trypsinized, washed, and resuspended in serum-free IMEM medium at a concentration of 10^8 cells/ml. Aliquots of 10 μl were injected into the cleared inguinal mammary fat pad of 3 week-old female BALB/c mice (34). Eight weeks after transplantation, mice were sacrificed, and isografts were fixed in 10% formalin in 1× PBS, embedded in paraffin, and stained with H&E by the Histopathology Core Facility, Lombardi Cancer Center. In instances where COMMA-1D/PDK1 and COMMA-1D/PKCα cells produced adenocarcinomas, part of the tumor was removed aseptically and mechanically dispersed with a 28-gauge needle in 1× PBS. The suspension was centrifuged and replated in IMEM containing 1× supplement, 600 μg/ml G418 (Life Technologies, Inc.), and 50 μg/ml gentamicin. After four passages, cells were collected and cell extracts prepared in Buffer A for Western blotting with the myc-tag antibody. Histopathology was assessed by Dr. Baljit Singh, Histopathology and Tissue Shared Resource, Lombardi Cancer Center.

RESULTS

To evaluate the role of PDK1 in mammary epithelial cell transformation, mouse mammary epithelial cell line COMMA-1D was retrovirally transduced with PDK1 (Fig. 1). Cells expressing PDK1 exhibited a high degree of anchorage-independent growth in soft agar, a feature that is considered indicative of transformation.

To determine whether the activity of downstream substrates of PDK1 were changed, immunocomplex kinase assays were carried out (Fig. 2). Cells expressing PDK1 exhibited higher Akt1 activity as shown by enhanced phosphorylation of Thr308 (Fig. 2*A*). The activity of PKCα, as measured by autophosphorylation of Ser657, was increased almost 7-fold in response to IGF-I stimulation (Fig. 2*B*), but

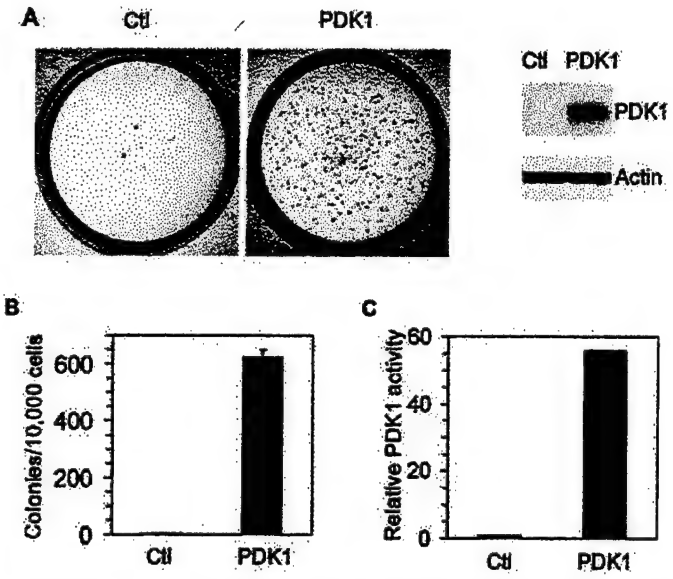

Fig. 1. Transformation of COMMA-1D cells by PDK1. *A*, COMMA-1D cells were retrovirally transduced with either *neo* (*Ctl*) or PDK1 (*PDK1*), and colony formation in soft agar was determined as the number of colonies per 10,000 cells plated. PDK1 and β-actin expression were determined in control (*Ctl*) and PDK1-expressing cells (*PDK1*) by immunoblotting with a myc-tag and β-actin antibody, respectively (*right*). *B*, quantitation of colony formation in soft agar as depicted in *A*. Each value is the mean of three experiments; *bars*, ±SD. *C*, PDK1 activity was determined in control (*Ctl*) and COMMA-1D/PDK1 cells (*PDK1*) as described in "Materials and Methods," and is expressed in arbitrary units relative to control cells transduced with the empty retrovirus (*Ctl*). Each value is the mean of three experiments.

the phosphorylation of another PDK1 substrate, S6K, was not changed (Fig. 2*C*).

To determine whether PDK1 altered the levels of PKCα or other PKC isoforms, cell lysates were analyzed by Western blotting (Fig. 3). The steady state level of PKCα was increased 6-fold, whereas other PKC isoforms remained unchanged (Fig. 3). PKCδ and θ were absent in these cell lines. We also noted that the steady state levels of Akt1, S6K, and SGK were not changed in COMMA-1D/PDK1 cells (Fig. 3).

Because COMMA-1D/PDK1 cells expressed greater Akt1 and PKCα activity, transformation was measured in COMMA-1D cells transduced with either Akt1 or PKCα (Fig. 4). In contrast to Akt1-expressing cells, which did not exhibit anchorage-independent growth in soft agar, PKCα-expressing cells showed significant growth, although less than PDK1-expressing cells (Fig. 4*A*). These results suggested that a portion of the transforming ability of PDK1 might be caused by the up-regulation of PKCα. When cells expressing PKCα were cotransduced with Akt1, cell transformation was increased by 20% in comparison to cells expressing PKCα alone (Fig. 4*B*), indicating a small degree of potentiation between the Akt1 and PKCα signaling pathways. When either Akt1 or PKCα was coexpressed with PDK1, no additional change in transformation occurred (Fig. 4*C*).

To determine the tumorigenicity of the various COMMA-1D cell lines, isografts of each cell line were transplanted into the cleared fat pad of syngeneic BALB/c mice, and the mammary gland was examined 8 weeks later (Fig. 5). Mice receiving isografts of COMMA-1D cells retrovirally transduced with either *neo* (Fig. 5*A*, *Ctl*) or Akt1 (Fig. 5*A*, *Akt1*) exhibited a normal ductal morphology. In contrast, all mice receiving isografts of COMMA-1D/PDK1 (Fig. 5*A*, *PDK1*) or COMMA-1D/PKCα (Fig. 5*A*, *PKCα*) cells presented with poorly differentiated mammary adenocarcinomas. PDK1-derived tumors exhibited an acinar morphology with an absence of glandular structure and were highly invasive to the musculature, as well as highly vascular (results not shown). PKCα-derived tumors were more focal,

not as invasive, and exhibited a squamous morphology. To ascertain the origin of the tumors, a portion of the tumor was removed and cultured *in vitro* in the presence of G418. PKCα and PDK1 expression in the respective COMMA-1D cell lines used for the isografts were comparable with their levels expressed in the cell lines derived from these tumors (Fig. 5*B*, *C*).

PDK1 expression was also evaluated in several human breast cancer cell lines by Western blotting (Fig. 6). In all instances, PDK1 levels were greater in the cancer cells compared with untransformed human mammary epithelial cell line MCF-10A. It was also noted that all of the cell lines expressed variable levels of a M_r 60,000 form in addition to the M_r 63,000 form of PDK1 and may represent an NH_2-terminally truncated PDK1.

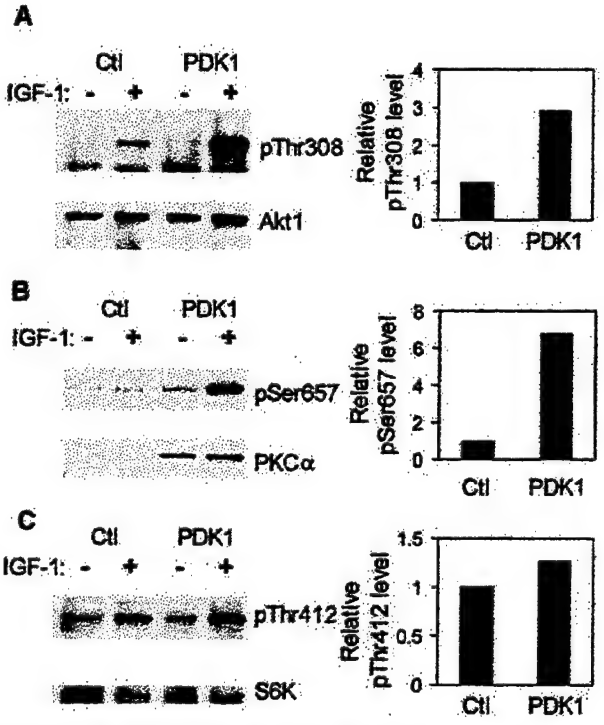

Fig. 2. Akt1 and PKCα activity, but not S6K activity are increased in COMMA-1D/PDK1 cells. *A*, COMMA-1D cells were retrovirally transduced with either *neo* (*Ctl*) or PDK1 (*PDK1*), serum-starved overnight, and then grown in the absence (−) or presence (+) of 100 ng/ml IGF-I. Akt1 was immunoprecipitated from cell lysates, and pThr308Akt1 and Akt1 were measured by immunoblotting. The *bar graph* represents the net increase in pThr308 produced by IGF-I normalized to the level of Akt1. *B*, cell lysates were prepared as described in *A*, PKCα was immunoprecipitated, and pSer657PKCα and PKCα were measured by immunoblotting. The *bar graph* represents the net increase in pSer657 normalized to the level of PKCα. *C*, cell lysates were prepared as described in *A*, S6K was immunoprecipitated, and pThr412S6K and S6K were measured by immunoblotting. The *bar graph* represents the net increase in pThr412S6K normalized to the level of S6K.

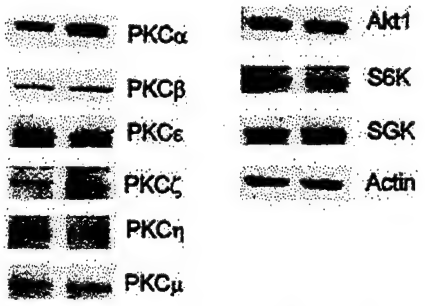

Fig. 3. PKC, Akt1, S6K, and SGK levels in COMMA-1D cells. Cell lysates were prepared from COMMA-1D/PDK1 cells, and protein kinase levels were analyzed by Western blotting as described in "Materials and Methods."

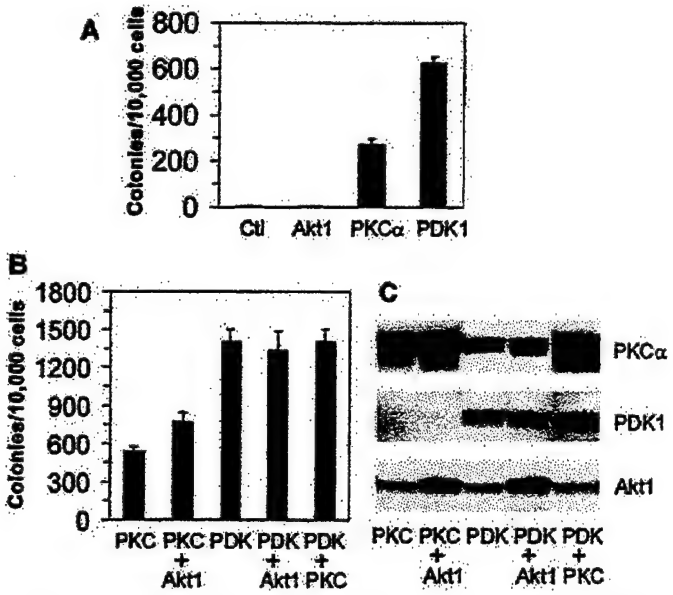

Fig. 4. Akt1 enhances PKCα-mediated transformation of COMMA-1D cells. *A*, COMMA-1D cells were retrovirally transduced with *neo* (*Ctl*), Akt1 (*Akt1*), PKCα (*PKCα*), or PDK1 (*PDK1*). Colony formation in soft agar was determined as the number of colonies per 10,000 cells plated. Each value is the mean of three determinations; *bars*, ±SD. *B*, a second round of retroviral transduction was carried out in COMMA-1D/PKC cells with either *hyg* (*PKC*) or Akt1 (*PKC+Akt*), or in COMMA-1D/PDK1 cells with *hyg* (*PDK*), Akt1 (*PDK+Akt*), or PKCα (*PDK+PKC*). Colony formation in soft agar was determined as in *A*. Each value is the mean of three experiments; *bars*, ±SD. *C*, Western analysis of PKCα, PDK1, and Akt1 expression in the cell lines shown in *B*.

DISCUSSION

The purpose of this study was to determine whether activation of the PDK1 signaling pathway resulted in the transformation of mouse mammary epithelial cells. Our results indicate that wild-type PDK1 was highly transforming in mammary epithelial cells both in cell culture and as isografts in syngeneic mice. Although PDK1 expression by retroviral transduction was high by physiological standards, our findings that some human breast cancer cells also contain very high levels of PDK1 suggest that perhaps this signaling pathway is relevant to the malignant phenotype. Because PDK1 controls the activation of several protein kinase families, we wished to determine whether its known activation of Akt1 and PKCα (14, 15) was associated with its ability to produce transformation. The finding that PDK1 increased the steady state level of PKCα is consistent with the findings that PKCα is down-regulated in embryonic stem cells null for PDK1 (35). Whether PDK1 modulates PKC expression transcriptionally or enhances post-translational stability (35) remains to be established; however, we have noted that the human PKCα promoter (36) is activated in PDK1-expressing cells,[4] which may contribute to enhanced PKCα expression in this cell line. PDK1 can regulate PKCα activity directly through phosphorylation of its activation loop at Thr497 as well as its COOH terminus to allow autophosphorylation at Ser657 (11, 37, 38), and this is also consistent with our results in COMMA-1D/PDK1 cells. The present study also indicates that PKCα is oncogenic in its own right. Although, PKCα has not been found previously to transform mouse and rat fibroblasts *in vitro* or to be tumorigenic in xenografts of these cell lines (39, 40), ectopic expression of PKCα in either human mammary epithelial cell line MCF-10A (41) or mouse 3T3 fibroblasts (42) has been shown to increase anchorage-independent growth and motility that were reminiscent of a transformed phenotype (39, 41, 42).

[4] B-K. Park, X. Zeng, and R. I. Glazer, unpublished observations.

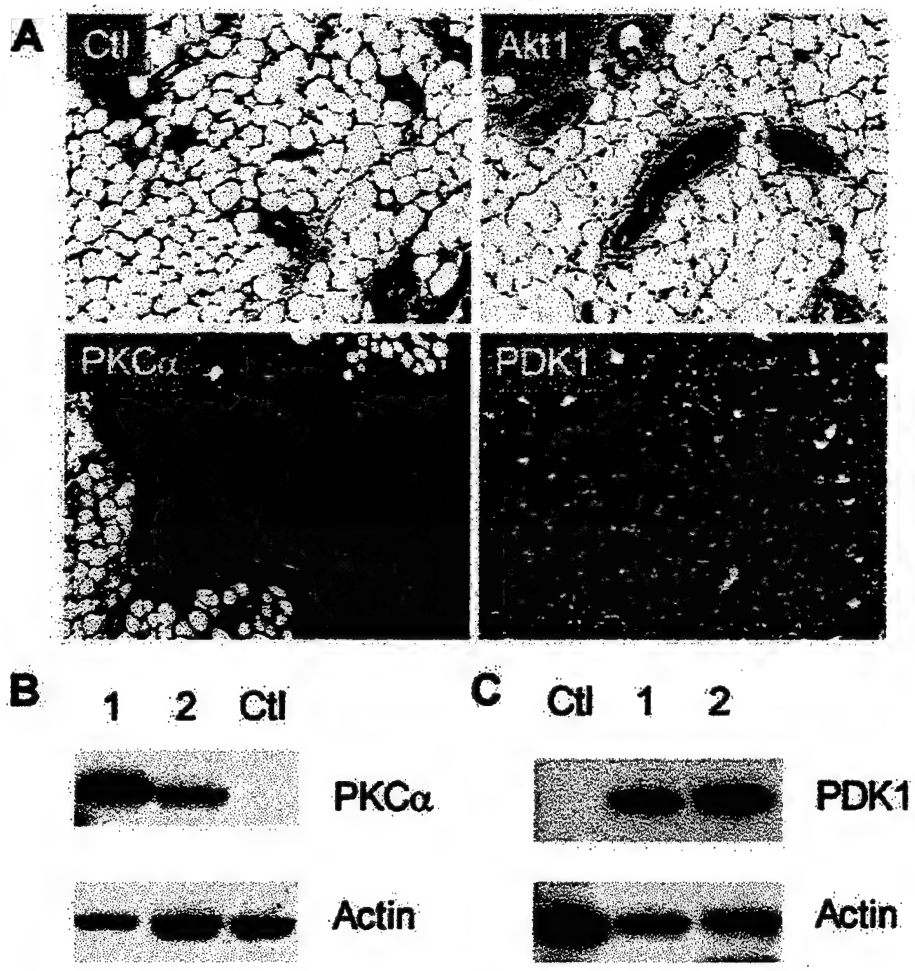

Fig. 5. Neoplastic transformation in isografts of COMMA-1D/PDK1 and COMMA-1D/PKCα cells. *A*, H&E-stained paraffin-embedded tissue obtained from isografts of COMMA-1D cells retrovirally transduced with *neo* (*Ctl*), Akt1 (*Akt1*), PKCα (*PKCα*), or PDK1 (*PDK1*). A representative section is shown for each tissue. Tumors derived from COMMA-1D/PKCα cells show an absence of glandular structure and a squamous morphology. Tumors derived from COMMA-1D/PDK1 cells show an acinar pattern with an absence of glandular structure. Magnification: ×100. *B*, Western analysis of PKCα expression in COMMA-1D/PKCα cells (*Lane 1*) and in a cell line derived from its respective tumor (*Lane 2*), and maintained in tissue culture. PKCα was analyzed by Western blotting for the myc tag. *C*, Western analysis of PDK1 expression in COMMA-1D/PDK1 cells (*Lane 1*), and in a cell line derived from its respective tumor (*Lane 2*) and maintained in tissue culture. PDK1 was analyzed by Western blotting for the myc tag.

Alternative signaling pathways other than those directly associated with PDK1 may also be involved in the regulation of PKCα and Akt1. Ha-Ras and polyoma middle T antigen activate PI3-K (43–45), and induce and activate PKCα (29, 46), as well as Akt1 activity (47, 48). Because PKCα is also an upstream activator of the Ras/Raf pathway (49), PKCα could conceivably regulate its own expression through the Ras pathway by a positive feedback loop. Coexpression of activated Ras and constitutively active Akt1 have also been shown to act synergistically to induce gliomagenesis *in vivo* (50). Because PKCα can directly stimulate Akt1 phosphorylation at Ser473 (51, 52), this could account for the small degree of potentiation of PKCα-mediated transformation by Akt1.

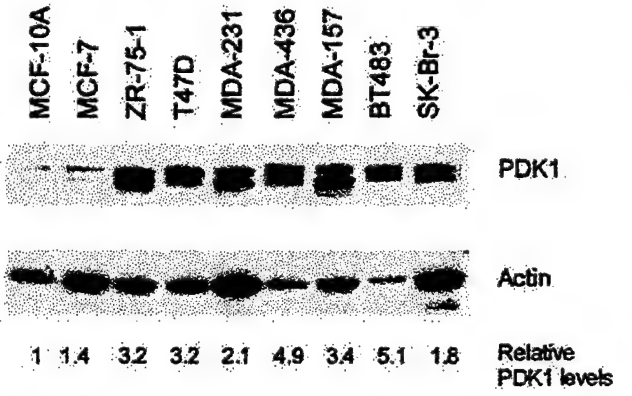

Fig. 6. PDK1 expression in human breast cancer cell lines. PDK1 levels in human breast cancer cell lines. Cell lysates were prepared, and Western blotting was carried out as described in "Materials and Methods." The relative level of PDK1 *versus* MCF-10A cells is denoted at the bottom of the figure.

One or more Akt isoforms highly expressed in human breast cancer cells are coupled to estrogen receptor and growth factor receptor activation (53–57). However, none of the Akt isoforms have shown oncogenicity in chick embryo and mouse 3T3 fibroblasts (30, 58–60), and mouse mammary epithelial cells (25), with the exception of constitutively activated forms of Akt (30, 59, 60). Moreover, mammary gland-directed expression of either constitutively active or wild-type Akt1 in transgenic animals has failed to induce a malignant phenotype (61–63), suggesting that additional factors or signaling pathways are required for transformation. The latter findings are consistent with the present study indicating that overexpression of Akt1 alone does not suffice to induce transformation or tumorigenesis.

The invasive characteristics of the tumors formed from allografts of COMMA-1D/PDK1 cells agree with our earlier finding that Akt1-expressing mammary epithelial cells exhibited increased extracellular matrix invasion through induction of MMP-2 (25). The acquisition of an invasive phenotype is also in accord with the findings that PKCα expression in MCF-7 breast cancer cells leads to enhanced anchorage-independent growth in soft agar (64), as well as increased motility and adhesion in human MCF-10A breast epithelial cells (41). Interestingly, isografts of COMMA-1D cells overexpressing v-Ha-Ras showed an undifferentiated morphology (65) that was very similar to the tumors arising from isografts of COMMA-1D/PDK1cells in the present study.

In summary, PDK1 expression in mouse mammary epithelial cells caused an increase in anchorage-independent growth in soft agar and

malignant transformation *in vivo*. Although, PDK1 up-regulated PKCα expression and Akt1 activity, only expression of PKCα was associated with transformation and tumor formation. These results suggest that the PKCα signaling pathway is a major component of the PDK1 oncogenic pathway.

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Transformation of Mammary Epithelial Cells by 3-Phosphoinositide-dependent Protein Kinase-1 Activates β-Catenin and c-Myc, and Down-Regulates Caveolin-1[1]

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ABSTRACT

3-Phosphoinositide-dependent protein kinase-1 (PDK1) plays a pivotal role in coupling growth factor receptor signaling to tumor cell proliferation, survival, and invasion. Protein kinase C (PKC) α, but not Akt1, was found previously to be downstream of PDK1-mediated transformation of mammary epithelial cells. To determine the basis for its oncogenic activity, signal transduction pathways mediated by PDK1 in mammary epithelial cells were investigated. β-Catenin/T-cell factor-dependent promoter activity was markedly activated in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells, which resulted in increased levels of the β-catenin/T-cell factor target genes *c-myc* and *cyclin D1*. In contrast, caveolin-1, of which the transcription is suppressed by c-myc, was down-regulated in PDK1- and PKCα-expressing, but not in Akt1-expressing cells. Analysis of 16 breast cancer cell lines established that caveolin-1 expression was either absent or reduced compared with breast epithelial cells, and that PDK1 was elevated in all of the cell lines. Interestingly, all of the cell lines known to be invasive expressed caveolin-1 to some degree, whereas, 5 of 6 cell lines that are not invasive did not express caveolin-1. Therefore, it appears that a concomitant gain of c-myc function and a loss or reduction of caveolin-1 are major determinants of PDK1- and PKCα-mediated mammary oncogenesis.

INTRODUCTION

PDK1[3] was first identified as a protein-Ser/Thr kinase that linked phosphatidylinositol 3′-kinase to Akt activation through growth factor-mediated signaling (1, 2). PDK1 contains an NH_2-terminal catalytic domain and a COOH-terminal pleckstrin homology domain (2), and is responsible for phosphorylating AGC kinases in their activation domain, a process that is essential for their full catalytic activity (3, 4). PDK1 activates a number of AGC kinases, including Akt (5, 6), PKC (7, 8), serum- and glucocorticoid-induced kinase (9, 10), and ribosomal p70S6-kinase (5, 11), implicating PDK1 as a pivotal signaling molecule in response to growth factors and metabolic effectors.

Akt, the most extensively studied PDK1 substrate, has been implicated in human cancers by promoting proliferation and survival, and inhibiting apoptosis (12, 13). Akt1 is the predominant isoform in most tissues and is highly expressed in breast cancer cells (14), as well as in primary breast cancer (15). PKCα also plays a role in tumor proliferation and survival as demonstrated by the antitumor activity produced by an antisense cDNA and antisense oligonucleotides (16, 17), as well as its oncogenic activity in mammary epithelial cells (18).

Apart from the similarities between Akt1 and PKCα in their proliferative and antiapoptotic functions, there are also clear differences in their tumorigenic potential. Akt1 was neither transforming *in vitro* (18) nor tumorigenic when expressed in the mammary gland of transgenic mice (19–21), whereas PKCα was highly oncogenic when overexpressed in mammary epithelial cells (18). Although both Akt1 and PKCα are downstream of PDK1, only PDK1 and PKCα exhibited transformation activity (18). To define the mechanistic differences between these protein kinases, they were overexpressed in mammary epithelial cells, and downstream signaling pathways were analyzed. The results of this study define for the first time an oncogenic pathway downstream of PDK1 and PKCα that is associated with increased c-Myc and cyclin D1 expression through β-catenin activation, as well as activation of the PKCα promoter and suppression of caveolin-1 expression. These data provide a unique framework for investigating the regulatory networks associated with PDK1-mediated transformation.

MATERIALS AND METHODS

Cells and Antibodies. Mouse mammary epithelial cell line COMMA-1D (obtained from Dr. Robert Dickson, Georgetown University, Washington, DC) and COMMA-1D cells expressing Akt1, PDK1, PKCα, or vector control (18) were maintained at 37°C under 5% CO_2 in IMEM supplemented with 2.5% fetal bovine serum, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin. Human breast cancer cell lines MDA-MB-157, BT20, BT549, MCF-7/ADR, MDA-MB-468, BT474, Hs578T, MCF-7/Ras, MDA-MB-231, MDA-MB-436, BT483, ZR75-1, SK-Br-3, MCF-7, MDA-MB-435, and T47-D, and human mammary epithelial cell line MCF-10A were obtained from the Lombardi Cancer Center Tissue Culture Core Facility. HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA), and HAB85 cells have been described previously (22). Monoclonal antibodies to cyclin D1, c-Myc, β-actin, and rabbit anti-caveolin-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-caveolin-1 was obtained from BD Biosciences Co. (San Diego, CA). Biotinylated goat antirabbit IgG, ABC reagent, and diaminobenzidine were purchased from Vector Laboratories (Burlingame, CA).

Plasmids. Plasmids were obtained from the following sources: β-catenin/TCF luciferase (TopFlash) from Upstate Biotechnology (Lake Placid, NY), c-Myc promoter luciferase plasmid pDel-1 from Dr. Burt Volgelstein, Johns Hopkins University (Baltimore, MD), TK-*Renilla*-luciferase from Promega (Madison, WI), PKCα reporter plasmid −1571/+77 as described (23), human myc-tagged PDK1 in pCMV5 from Dr. Dario Alessi, University of Dundee (Dundee, United Kingdom), human Akt1 in pTarget as described (14), and human c-Myc in the RCAS viral vector from Dr. Yi Li, Memorial Sloan-Kettering Cancer Center (New York, NY). c-Myc was amplified by PCR and subcloned into pCR3.1-TA (Invitrogen Corp., Carlsbad, CA) using the forward and reverse primers, 5′-GCC ACC ATG CCC CTC AAC GTT and 5′-CC TTA CGC ACA AGA GTT CCG, respectively.

Western Blotting. Total cell lysates from 2×10^5 cells were prepared by lysing the washed cell pellet directly in Laemmli sample buffer and boiling for 10 min. Caveolin-1 was extracted in lysis buffer containing: 1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 60 mM octylglucoside, and a protease inhibitor mixture (Boehringer-Mannheim Co., Indianapolis, IN) for 45 min at 4°C. Lysates were clarified by centrifugation at 13,000 × *g* for

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[3] The abbreviations used are: PDK1, 3-phosphoinositol-dependent protein kinase-1; PKC, protein kinase C; TCF, T-cell factor; IMEM, improved minimal essential medium; GFR, growth factor receptor; IGF-1, insulin-like growth factor-1; PAGE, polyacrylamide gel electrophoresis; PH, preckstrin homologue; P13K, phosphoinositide 3-kinase; GSK, serum- and glucocorticoid-induced kinase.

[4] Z. Xie, and R. I. Glazer, unpublished observations.

15 min at 4°C, and mixed with 10× Laemmli sample buffer and boiled. Lysates were separated by SDS-PAGE in 10% polyacrylamide gels, blotted onto nitrocellulose, and analyzed by Western blotting with the antibodies described.

Immunostaining. Cells were seeded on cover slips in a six-well plate overnight, and fixed with 4% formaldehyde in PBS for 30 min after removing the medium. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked in 2% goat serum in PBS for 30 min, and washed three times in PBS. Slides were stained with rabbit anti-caveolin-1 antibody in blocking buffer for 1 h, washed three times with PBS, and incubated with secondary biotinylated goat antirabbit IgG for an additional hour. Antigen was visualized using ABC Vectastain and diaminobenzidine as substrate. Slides were counterstained with Harris-modified hematoxylin (Fisher Scientific, Pittsburgh, PA) and mounted in Permount.

Luciferase Assay. Cells were seeded in a 24-well plate at a density of 40,000 cells/well overnight. Cells were then transfected with 100 ng TopFlash, 50 ng pDel-1/c-Myc, or 50 ng p[-1571/+77]PKCα promoter, and 5 ng TK-*Renilla*-luciferase (Promega) using either Effectene (Qiagen Inc., Valencia, CA) or Lipofectamine Plus reagent (Invitrogen Corp.) according to the manufacturer's instructions. In some experiments, reporter plasmids were cotransfected with 50 ng of pTarget/Akt1, pCMV5/PDK1, pCR3.1/c-Myc, or the empty vector. Transfection efficiency was monitored by transfection with 100 ng pEGFP-C1 (BD Biosciences, Palo Alto, CA); ≥50% efficiency was observed in all of the cell lines. Luciferase activity was measured 24 h after transfection using the Luciferase Assay System (Promega), normalized to *Renilla*-luciferase activity, and is expressed as the activity relative to vector-transfected controls.

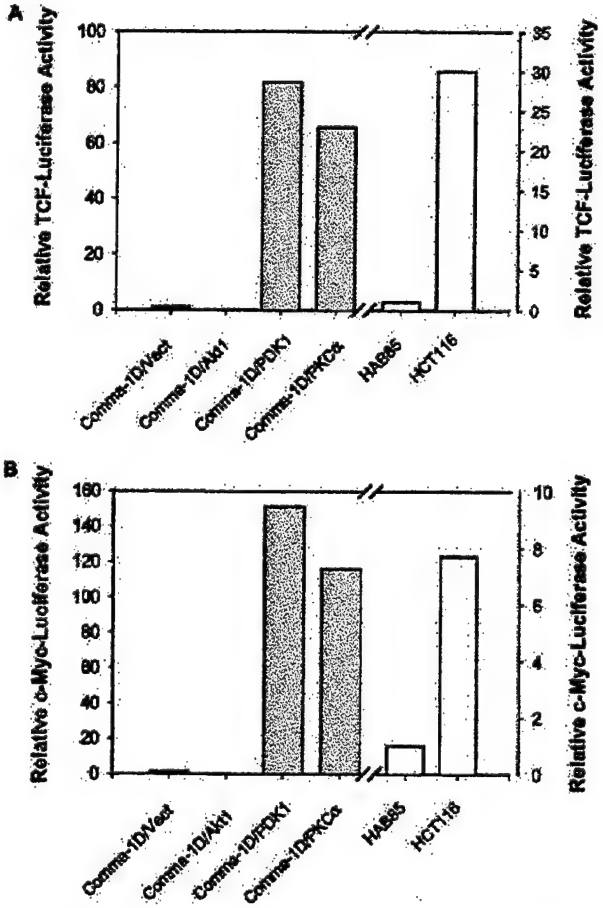

Fig. 1. β-Catenin/TCF reporter activity in PDK1- and PKCα-expressing cells. *A*, vector-, Akt1-, PDK1-, or PKCα-expressing COMMA-1D cells were transfected with a β-catenin/TCF luciferase plasmid and a *Renilla*-luciferase plasmid to correct for efficiency, and luciferase activity was measured 24 h later. Each value represents the mean of three experiments that have been normalized to *Renilla*-luciferase activity, and is expressed as activity relative to control cells; *bars*, ±SE. HAB85 cells deficient in a mutated, activated β-catenin allele and parental HCT116 cells are shown for comparison. *B*, cells were transfected with the pDel-1/c-Myc promoter luciferase plasmid, and luciferase activity was measured 24 h later. Each value represents the mean of three experiments, and is expressed as activity relative to control cells. HAB85 and HCT116 cells are shown for comparison.

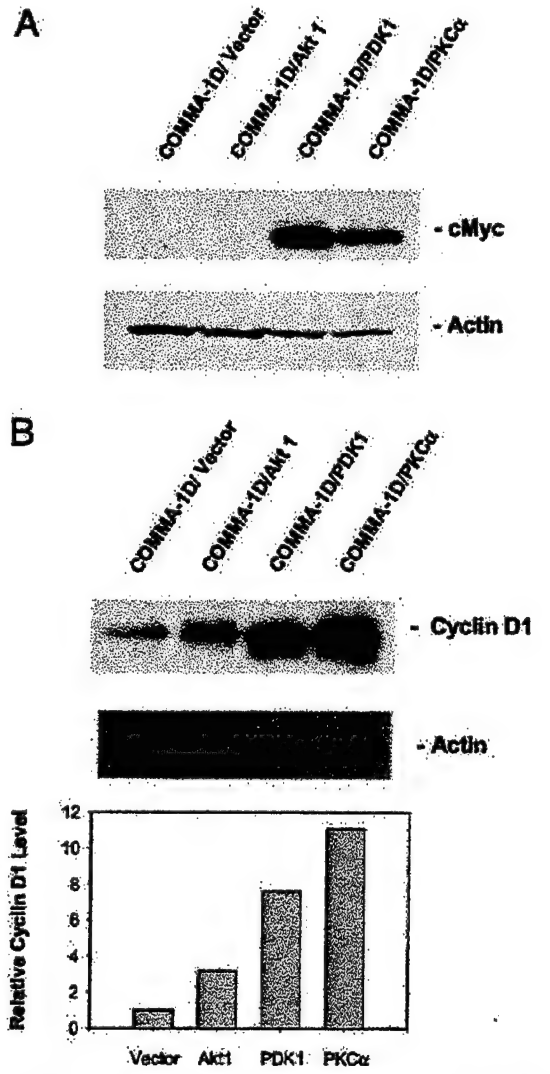

Fig. 2. c-Myc and cyclin D1 levels in PDK1- and PKCα-expressing cells. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting. *A*, c-Myc levels. Blots were probed with a c-Myc antibody, stripped, and reprobed with a β-actin antibody. *B*, cyclin D1 levels. Blots were probed with a cyclin D1 antibody, stripped, and reprobed with a β-actin antibody. *Bar graph*, relative cyclin D1 levels normalized to β-actin determined by densitometric scanning of *B*. Results are the mean of two independent experiments.

RESULTS

One oncogenic pathway suggested to be downstream of PDK1 is the β-catenin/TCF pathway. Activation of the Wnt1/β-catenin pathway in the mammary gland is highly oncogenic (24), and GSK-3β, which suppresses β-catenin function, is inhibited downstream of PDK1 (13, 25). To assess the activity of this pathway in our cell lines, cells were transfected with a TCF-dependent promoter luciferase plasmid (26). β-Catenin/TCF reporter activity was markedly increased by ~80- and 65-fold, respectively, in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells (Fig. 1*A*). HAB85 cells heterozygous for the wild-type *β-catenin* allele and deficient in the mutated, oncogenic allele (22) were included as controls for the specificity of the assay. β-Catenin/TCF-dependent activity was markedly suppressed in HAB85 cells in comparison with parental HCT116 cells containing the oncogenic allele (Fig. 1*A*).

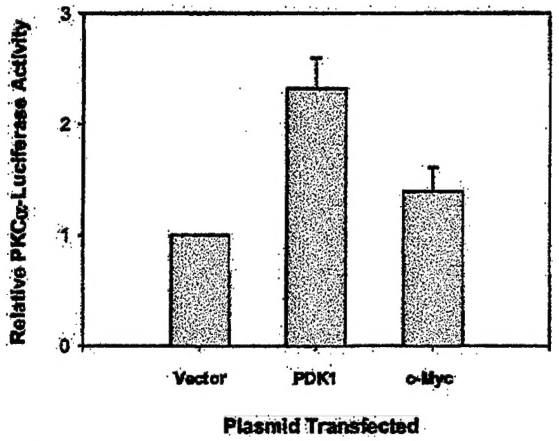

Fig. 3. PKCα promoter activity in PDK1-expressing cells. Cells were cotransfected with the p[-1571/+77]PKCα promoter luciferase plasmid, and a control, c-Myc, or PDK1 plasmid, and luciferase activity was measured 24 h later. Each value represents the mean ± SEM of three experiments and is expressed as activity relative to control cells.

The β-catenin target, c-Myc (27), which is oncogenic in the mammary gland (28), was next evaluated using a c-Myc promoter reporter gene (Fig. 1*B*). c-Myc promoter activity was markedly increased in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells; promoter dependence on β-catenin activation was again absent in HAB85 cells lacking the oncogenic *β-catenin* allele (Fig. 1*B*).

To additionally characterize the downstream effectors of the β-catenin pathway operative in PDK1- and PKCα-expressing cells, levels of the β-catenin targets, c-Myc and cyclin D1 (29), were determined. c-Myc was undetectable in control and Akt1-expressing cells, but was increased significantly in PDK1- and PKCα-expressing cells (Fig. 2*A*). Levels of cyclin D1 were also much greater in PDK1- and PKCα-expressing cells than in control or Akt1-expressing cells (Fig. 2*B*). Cyclin D2 was not detectable (4), although it has also been suggested to be the mediator of c-Myc-induced mammary tumorigenesis (30).

We found previously that PKCα was up-regulated in PDK1-expressing cells (18). To determine whether this was linked to c-Myc expression, a PKCα promoter reporter plasmid (23) that contains several c-Myc response elements was tested (Fig. 3). Although, PDK1 increased PKCα promoter activity 2.3-fold, coexpression of c-Myc did not markedly enhance promoter activity, suggesting that additional transcription factors likely contribute to regulating PKCα transcription downstream to PDK1.

To additionally delineate the role of c-Myc in PDK1-mediated oncogenesis, expression of the tumor suppressor caveolin-1 was determined. Caveolin-1 is the principal component of plasma membrane caveolae that are involved in sequestering several signal transduction effectors (31, 32). Caveolin-1 functions as a tumor suppressor gene in breast cancer (33), and its transcription is suppressed by c-Myc (34, 35). In PDK1- and PKCα-expressing cells, caveolin-1 expression was undetectable, although it remained unchanged in Akt1-expressing cells (Fig. 4*A*). Immunostaining for caveolin-1 also indicated clear differences among control, Akt1-, PKCα-, and PDK1-expressing cells, where it was undetectable in the latter two cell lines (Fig. 4*B*).

To examine the relationship between PDK1 and caveolin-1 expression, 16 breast cancer cell lines and 1 breast epithelial cell line were analyzed by Western blotting (Fig. 5*A*). Caveolin-1 was either reduced or undetectable in most cell lines, and all of the cell lines expressed 1.4–5-fold higher PDK1 levels in comparison with MCF-10A cells (Fig. 5*B*). Interestingly, 7 of 7 cell lines known to be invasive expressed caveolin-1, whereas 5 of 6 cell lines known not to be invasive did not express caveolin-1 (Fig. 5*B*). The invasive status of 3 cell lines is unknown.

DISCUSSION

PDK1 and its downstream effector, PKCα (36), were shown previously to mediate mammary epithelial cell transformation and tumorigenesis (18). Akt1, a well-characterized PDK1 substrate, did not exhibit such activity. The present study now suggests that activation

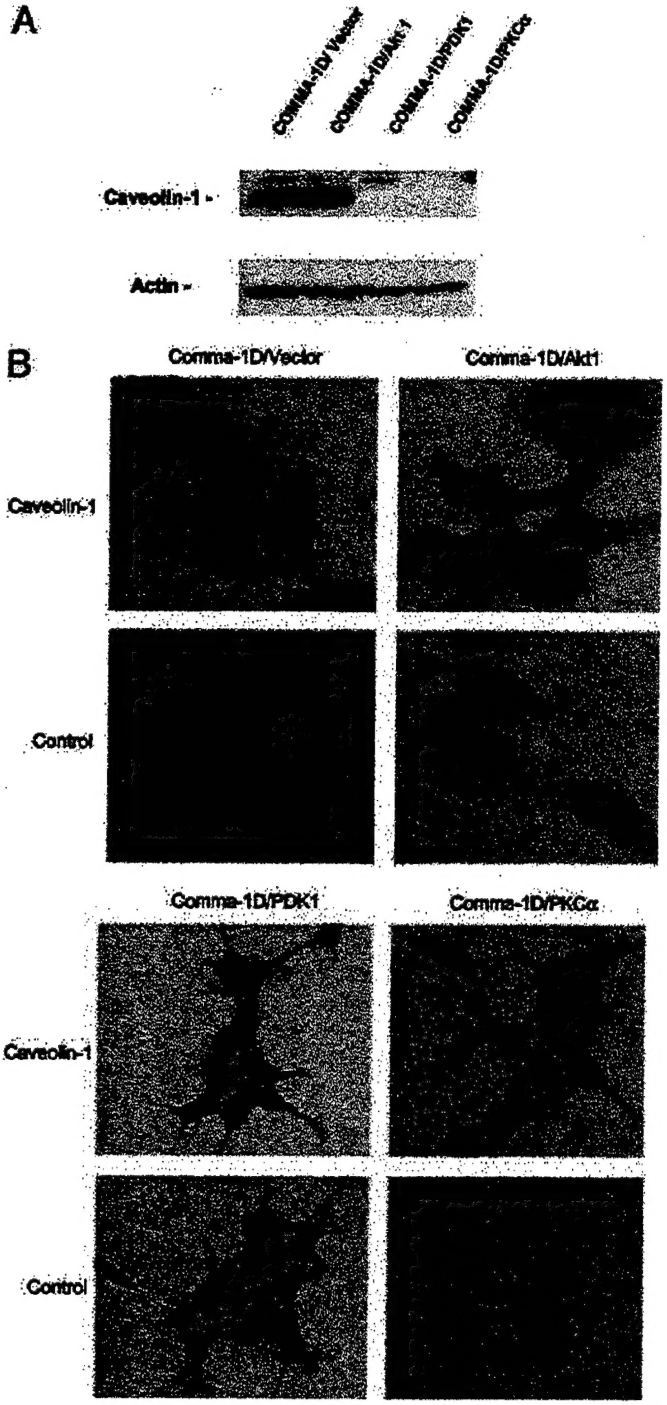

Fig. 4. Caveolin-1 expression in PDK1- and PKCα-expressing cells. *A*, cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with an anti-caveolin 1 antibody. Blots were stripped and reprobed for β-actin. *B*, cells were fixed and stained with an anti-caveolin-1 antibody, and antigen was detected by peroxidase staining. Magnification ×400.

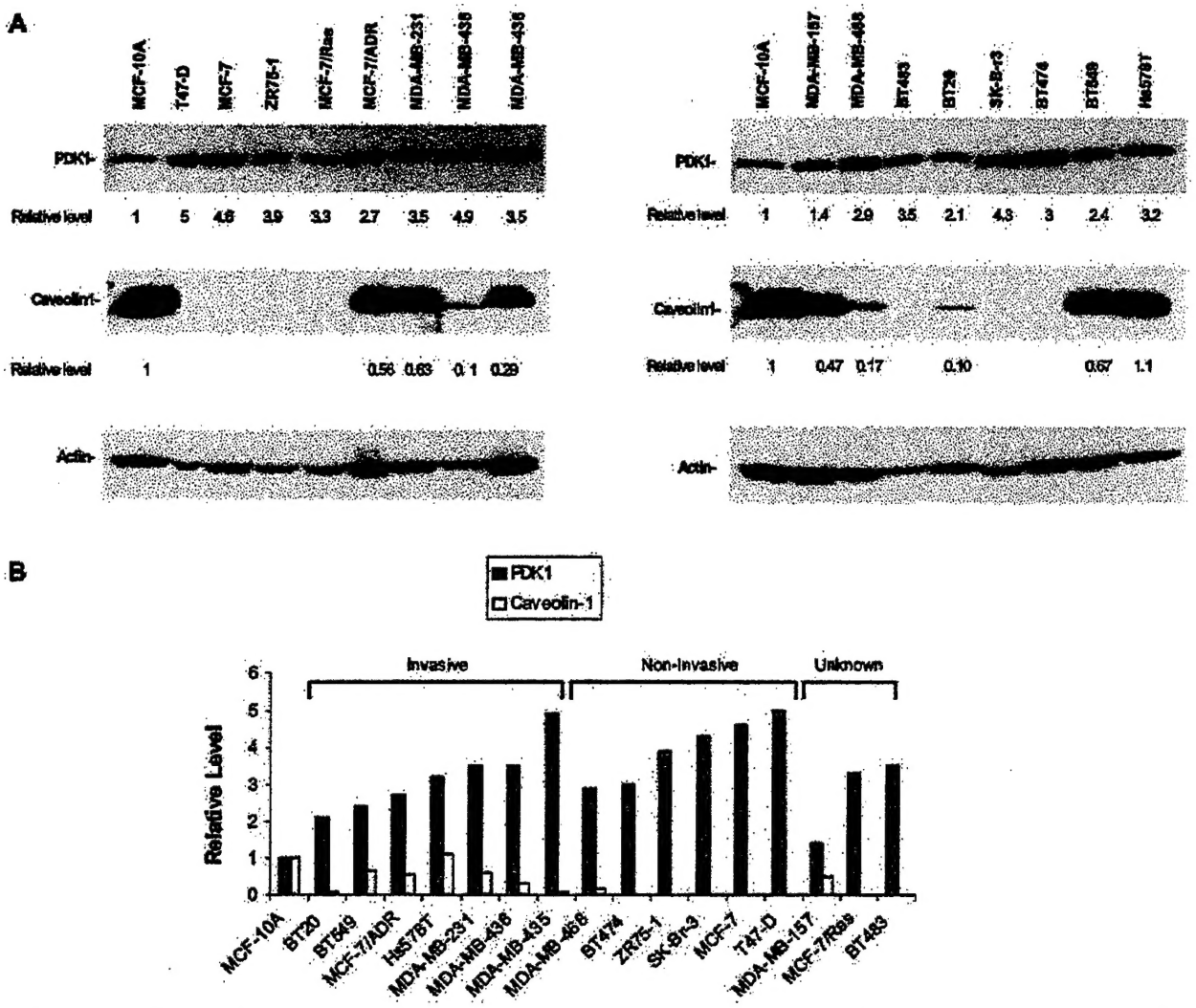

Fig. 5. Caveolin-1 and PDK1 expression in human breast cancer cell lines. *A*, cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with an anti-caveolin-1, anti-PDK1, or antiactin antibodies. The values listed below each *lane* are the level of caveolin-1 or PDK1 normalized to β-actin levels relative to MCF-10A cells. *B*, PDK1 and caveolin-1 levels relative to MCF-10A cells. Each point is based on the data in *A* where PDK1 and caveolin-1 levels in MCF-10A were set to 1.

of β-catenin/TCF and two of its target genes, c-Myc and cyclin D1, correlate with the oncogenic potential of PDK1 and PKCα in mammary epithelial cells, and that activation of this signaling axis results in the down-regulation of the tumor suppressor, caveolin-1 (Fig. 6). These changes appear to explain the lack of oncogenicity of both wild-type and constitutively active Akt1 in the mammary gland, despite its ability to block apoptosis, increase cyclin D1 levels, and cause hyperplasia (19–21). However, the lack of Akt1 oncogenicity in the mammary gland may not pertain to all of the Akt isoforms and target tissues, because expression of lymphocyte-targeted Akt2 caused lymphomas (37).

c-Myc up-regulation occurred downstream of PDK1 and PKCα, but not Akt1, and the expression of this oncogene correlated with the relative oncogenic activity of PDK1 and PKCα in mammary epithelial cells (18), and the known oncogenic activity of c-Myc in fibroblasts (38, 39) and the mammary gland (28). However, c-Myc activation did not appear to explain the up-regulation of PKCα occurring in PDK1-expressing cells, because it had only a limited effect on PKCα promoter activity (Fig. 3). This suggests that other transcription factors (23) or post-translational processes modulated by PDK1 are likely to be involved.

Activation of the TCF coactivator, β-catenin, is associated with transformation in many tissues (40, 41). This is particularly striking in the mammary gland where expression of a constitutively active β-catenin transgene resulted in rapid tumorigenesis and elevated cyclin D1 expression (42). In the present study, it is less clear how elevated cyclin D1 levels found in PDK1- and PKCα-expressing cells relate to transformation, because Akt1 also increased cyclin D1 levels, albeit to a lesser extent. Because Akt1 expression in the mammary gland was not tumorigenic (19), and the phenotype of mouse mammary tumor virus-cyclin D1 mice is not strongly tumorigenic (43), the contribution of cyclin D1 to transformation may be more quantitative than qualitative or require a secondary event such as tumor suppressor down-regulation. More than 50% of human breast cancers overexpress cyclin D1 (44, 45), and cyclin D1 is required for Neu- and Ras-mediated transformation, but not for c-Myc- and Wnt1-induced mammary tumorigenesis (30). Although, Akt1 has a key role in cell survival (13, 14, 46) and induces cyclin D1 expression in the mammary gland (19), its lack of oncogenicity (18) suggests that cyclin D1 may be necessary but insufficient for transformation through the PDK1 signaling pathway.

Although the β-catenin/c-Myc pathway activates a number of proto-oncogenes (47, 48), c-Myc also serves to down-regulate the tumor suppressor, caveolin-1, by inhibiting initiator element function in the caveolin-1 promoter (34, 35). This was readily apparent in PDK1- and PKCα-expressing cells where caveolin-1 was absent, and

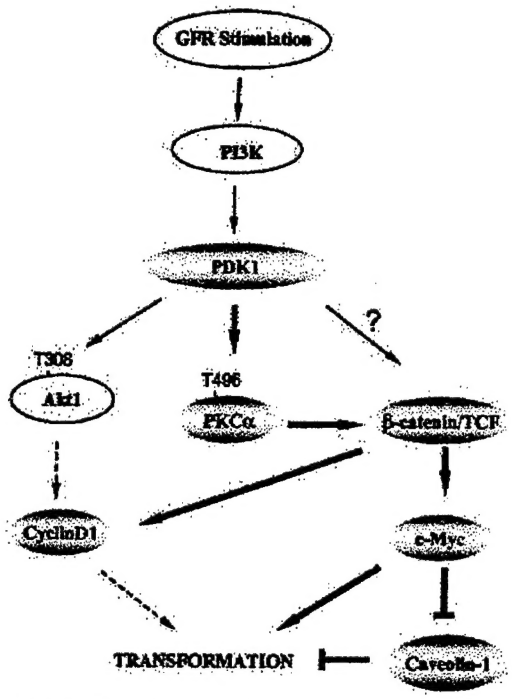

Fig. 6. PDK1 transformation pathway in mammary epithelial cells. Growth factor receptor (*GFR*) stimulation activates phosphatidylinositol 3′-kinase to generate phosphatidylinositol-3,4,5-trisphosphate, which recruits PDK1 to the plasma membrane. PDK1 phosphorylates Akt1 and PKCα at T308 and T496, respectively, to prime catalytic activity. PDK up-regulates PKCα expression and activates the β-catenin pathway; it is uncertain whether PDK1 directly activates β-catenin. β-Catenin activates cyclin D1 and c-Myc transcription, and c-Myc suppresses caveolin-1 transcription. Increased c-Myc activity and loss of caveolin-1 lead to transformation. *Solid bold arrow*, marked activation; *solid arrow*, activation; *dashed arrow*, weak activation; *blunt line*, inhibition.

correlated inversely with c-Myc and PDK1 expression. Caveolin-1 has been proposed to function as a scaffolding protein to bind and sequester signaling molecules such as PKCα, c-Src, eNOS, and G proteins in caveolar membrane rafts, where their activity is inhibited (31, 49). The suppressor effect of caveolin-1 on malignant transformation has been illustrated by its ability to reverse H-Ras- and v-Abl-induced transformation in fibroblasts (50), as well as the tumorigenicity of MCF-7 breast cancer cells (51). In the present study, cell morphology was also markedly changed in PDK1- and PKCα-expressing cells, which suggests changes in the actin cytoskeleton, such as those observed in 3T3 cells expressing an oncogenic caveolin-1 mutant (33). Thus, loss of caveolin-1 expression by PDK1 and PKCα likely plays a key role in their ability to mediate mammary epithelial cell transformation.

The low levels or absence of caveolin-1 and the high expression of PDK1 in all 16 of the breast cancer cell lines examined is of great interest, because it implies a functional link between these proteins. A tumor suppressor role for caveolin-1 is suggested by its absence in mammary tumors in mouse mammary tumor virus-c-Myc, -Neu, -Src, and -Ha-Ras transgenic mice (52), as well as the presence of a transforming P132L caveolin-1 mutation in 16% of highly aggressive scirrhous breast cancers (33). However, studies in primary prostate and pancreatic cancer indicate just the converse, *i.e.*, that positive caveolin-1 expression is associated with tumor progression and poor clinical outcome (53, 54). Our results identified a subset of breast cancer cell lines that have been characterized previously as invasive (55–57), and that express caveolin-1 and PDK1. The association of caveolin-1 mRNA expression with invasiveness in MDA-MB-435, BT549, MDA-MB-231, and Hs578T cells was also noted previously (58). The reappearance of caveolin-1 in drug-resistant MCF-7/ADR cells (59), and its association with P-glycoprotein (60), indeed suggests a role for caveolin-1 in pleiotropic drug resistance and tumor progression.

In summary, the present study demonstrates that PDK1 and PKCα, but not Akt1, results in activation of β-catenin-mediated c-Myc and cyclin D1 expression that is associated with the down-regulation of caveolin-1. The concomitant gain of c-Myc function and loss of caveolin-1 function suggest they are major determinants of PDK1- and PKCα-mediated mammary oncogenesis.

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